SOME GENETIC AND DEVELOPMENTAL STUDIES IN THE SPECIES *SCLEROSTACHYA FUSCA*, A. CAMUS AND *NARENGA PORPHYROCOMA* (HANCE) BOR, AND THEIR HYBRIDS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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We certify that we have read this thesis and that in our opinion it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Agronomy.

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INTRODUCTION

Plant materials used in this study include the intergeneric hybrids and their parents from Narenga Bor and Sclerostachya (Anderss.) A. Camus. These genera are related to the genus Saccharum L. of the tribe Andropogoneae Dumort. of the family Gramineae A. L. de Jussieu. The first species, Narenga porphyrocoma (Hance) Bor, was not separated from the genus Saccharum until 1940. Later its generic characteristics were widened to include other grasses including Erianthus chrysothrix Hack. The second species used in this study was Sclerostachya fusca (Roxb.) A. Camus.

Narenga and Sclerostachya cross readily with each other and with complete pairing of the chromosomes (Raghavan, 1951). Saccharum officinarum L. will cross easily with both genera. This is believed to be due to its high polyploidy and the subsequent autosyndesis that frequently follows (Janaki-Ammal, 1941; Raghavan, 1951). In these crosses it was found that the haploid gametes of the parents functioned, which indicated the existence of some chromosome homology.

The objective of this study was to investigate the genetic behavior of the two species and their hybrids by means of zone electrophoresis. In applying such a technique there is a possibility of finding a new approach to solve certain problems encountered in intergeneric hybrids. One of these problems is their identification as true hybrids. In a group of plants like the ones investigated there is a need for identifying the hybrids because of pollen contamination, male sterility, polyembryony, and sexual parthenogenesis (Grassl, 1962).

The success or failure of the application of a technique such as zone electrophoresis in studying intergeneric hybrids from Narenga and Sclerostachya can be related to other cases, particularly where sugar cane is involved. This is true because of the success achieved in sugar cane breeding by the inclusion of genes from the wild canes in the commercial varieties.

In recent work, Urata and Rotar (unpublished), established that callus tissue could be produced from inflorescences from grass species and plants can be formed. A project was undertaken to study the possible changes in enzyme systems brought about by changes in the different developmental stages; inflorescences, callus, and plants. Because of the claim that the peroxidase enzyme system interacts with plant growth hormones (Galston and Dalberg, 1954; Boll, 1965), investigation was made of the peroxidase enzyme systems obtained from callus tissue grown in different culture media.

LITERATURE REVIEW

Peroxidase Isozyme Variation:

In sugar cane breeding, intergeneric hybridization and its utilization in the breeding program is one of the few means available for achieving continuing progress. Species from six genera; Erianthus Michx, Miscanthidium, Miscanthus, Narenga Bor, Sclerostachya (Hance) A. Camus and Sorghum Moench have been successfully hybridized with the genus Saccharum L. (Grassl, 1962). Intergeneric hybridization in sugar cane becomes very important since there is the opportunity for transference of chromosomes and finally their desired segments or genes into commercial varieties (Grassl, 1962).

Very little is known about the genetics of N. porphyrocoma and S. fusca. Janaki-Ammal (1940) reported that the chromosome number in S. fusca was 48 and 96 in plant collections from two different sites in India. Bremer (1923) reported that N. porphyrocoma has 30 chromosomes, this was verified by Janaki-Ammal (1941). Dr. Peter P. Rotar, Associate Professor in Agronomy, Department of Agronomy and Soil Sciences, University of Hawaii (personal communication), found that the chromosome number in both N. porphyrocoma and S. fusca grown at the Hawaiian Sugar Planters' Association was thirty. The basic chromosome number is believed to be five with a complex degree of polyploidy.

The concept that protein synthesis is genetically controlled lends itself as an indirect means for studies in genotypic variation (Allison, 1959; Ashton, 1965). The development of horizontal zone electrophoresis by Smithies (1955) and its subsequent modifications and improvement in recent years has produced very interesting results. Considerable use

has been made of the fact that enzymes and proteins, in general follow mendelian ratios in the material studied by many workers (Beckman and Johnson, 1964; Ashton, 1965).

Results are not always clear-cut. Since the one gene, one enzyme theory was presented a great number of postulates have been offered to explain conflicting results. Differential gene action has been suggested by some researchers (Siegel and Galston, 1967). Others have suggested the existence of an enzyme in a dimer or specific multiple form of a polypeptide chain in certain enzymes and proteins (Burns and Johnson, 1967). According to this, two genetic pieces of information, i.e. genes can give rise to a multiple number of enzymes. Repression and induction of certain enzymes and proteins have been established (Jacob and Monod, 1961). This led to numerous investigations in the gene itself as the basic unit of inheritance. In general, two types of genes are postulated to exist. The first is the structural gene unit, which is directly involved in the production of a certain type of a polypeptide chain with amino acids arranged in specific order; the second is the regulator gene which controls the amount of the enzyme to be synthesized (Dixon and Webb, 1964). These two genes may be in different chromosomes but are usually found closely adjacent to each other in the same chromosome (Watson and Crick, 1953).

Difficulties in isozyme studies may arise from their behavior during electrophoretic separation. In peroxidase isozyme studies the existence of "shadow bands" under conditions of rapid separation has been suggested (Dr. Barbara Siegel, Assistant Professor of Biology, University of Hawaii, personal communication). This phenomenon may be

explained on the basis that the enzyme in its rapid migration through the gel is subjected to a certain degree of resistance. This may result in the breakdown of the giant molecules into fragments, allowing them to migrate faster and to produce faint, short bands which become more apparent as the distance from the origin increase.

Enzyme-Substrate Reactions of Peroxidases in Relation to Biochemical Assays:

Horse-radish peroxidase was first crystallized by Theorell (1942). Since then, the molecular heterogeneity of this enzyme has been established (Jermyn, 1952; Klapper and Hackett, 1965; Shannon, et al., 1966). The enzyme is a group of haemoproteins with one prosthetic group per molecule (Dixon and Webb, 1964). The peroxidases are specific for hydrogen peroxide although they use a wide range of substances as hydrogen donors (Theorell, 1951). A number of inhibitors for these enzymes were found; sulfides, fluorides and hydroxylamine. Mn^{++} ions are claimed to be necessary for certain systems of the enzyme substrate reaction (Akazawa and Conn, 1958).

Four distinct complexes have been observed in the enzyme substrate reaction (Chance, 1949). Complex I, a green compound formed on the addition of hydrogen peroxide, is highly unstable and rapidly converts to complex II. Complexes III and IV, red and more stable compounds, are formed on the addition of excess hydrogen peroxide (Chance, 1949). Dixon and Webb (1964) present the overall reactions, postulated by Chance as follows:

$$\text{I} \quad E + H_2O_2 \rightleftharpoons E\,S_1$$

$$\text{II} \quad E\,S_1 + AH_2 \xrightarrow{K+2} E\,S_{11} + AH$$

$$\text{III} \quad E\,S_{11} + AH \xrightarrow{K+3} E + -AH$$

$$2\,-AH \longrightarrow A + AH$$

where E stands for the enzyme, S for the substrate and AH_2 for the hydrogen donor.

There are two hypotheses as to the chemical nature of the substrates: (a) Chance (1949) believes Complexes I and II are in the following forms:

1. $Fep^{+++}\,(H_2O_2)$
2. $Fep^{+++}\,(OH.)$

The letter p stands for protein and Fe stands for iron. The general basis for this hypothesis rests on the idea that enzyme and substrate unite to form one compound which is then half-reduced in Complex II. (b) The other hypothesis, by George (1953), assumed that hydrogen peroxide oxidized the iron in the enzyme to a higher valency state. He gave the following equations:

$$\text{I} \quad Fep^{+++} + H_2O_2 \longrightarrow (FepO)^{+++} + H_2O$$

$$\text{II} \quad (FepO)^{+++} + AH_2 \longrightarrow (FepO)^{++} + -AH + H^+$$

$$\text{III} \quad (FepO)^{++} + AH_2 + H^+ \longrightarrow (Fep)^{+++} + -AH + H_2O$$

$$2\,-AH \longrightarrow A + AH_2$$

In the above equations another idea is introduced, the oxidation and reduction of the enzyme itself. Another complication was added when Yamazaki, et al. (1960) suggested the possibility of the formation

of intermediary radicals in these reactions.

It is clear that the enzyme-substrates are complex and unstable. Whether or not this instability is the direct cause of difficulties encountered in histochemical assays of the enzyme awaits further investigation. The conventional methods used in electrophoresis to stain the enzyme have not been very satisfactory (Jermyn and Thomas, 1954; Owen, et al. 1958). In the investigation report herein, a number of methods described by Jermyn and Thomas (1954) for the enzyme staining were attempted but most of the work was confined to the Benzidine test (Siegel and Galston, 1967). It was found that the benzidine blue faded very quickly causing the zymogram to blur and the storage and subsequent handling were unsatisfactory.

Isozyme Variations and Development:

The process by which explants, cultured in basal media with other complex nitrogenous substrates, develop and give rise to plants has been studied for a long time in dicotyledons (Steward and Shantz, 1956). Recently, Urata and Rotar, Department of Agronomy and Soil Science, University of Hawaii (unpublished) produced whole plants from callus tissue derived from young inflorescences of the family Gramineae. Urata and Rotar found that callus tissues grown in a media with 2,4-dichlorophenoxy acetic acid produced isozyme patterns different from those produced by callus tissue grown in a media lacking 2,4-D.

The peroxidase enzymes catalyze the oxidation of the plant growth hormone IAA (3-indole acetic acid) (Ockerse, et al., 1966). They are important in the regulation of growth and differentiation (Siegel and

Galston, 1967). Although nothing has been reported about actual enzyme activities in callus tissue derived from explants, their role in cells is important since 2 percent of proteins in plant cells are believed to be in the form of the enzyme. Their wide range of heterogeneity is believed to furnish biochemical protection against environmental stresses caused by a natural change in the habitat or by mutations (Siegel and Galston, 1967). The application of gibberellic acid to dwarf corn plants quantitatively altered several peroxidase isozymes systems (McCune, 1960). The interactions between enzymes and growth hormones are suggested to be directly involved in the regulation of plant growth (Siegel and Galston, 1967).

MATERIALS AND METHODS

Source of Plant Materials Used:

The following hybrids and parents of different clones of *N. porphyrocoma* and *S. fusca*, were studied (Table I).

Seeds from a cross within *N. porphyrocoma* were introduced from India in 1931 and germinated on Molokai by personnel of the Experiment Station of the Hawaiian Sugar Planters' Association (H.S.P.A.). One plant was obtained from this material. This plant was never given a number by the H.S.P.A. and has been identified only as *N. porphyrocoma*. The lack of an introduction and/or identification number has caused some difficulty in reporting on studies involving it and other clones of *N. porphyrocoma*. U.S. 62-1 is a cross within *N. porphyrocoma* made at the United States Department of Agriculture Sugar Cane Field Station, Canal Point, Florida. U.S. 62-2 is a cross within *S. fusca* made also at Canal Point, Florida. Seeds from the two crosses, introduced into Hawaii by the Genetic Department of H.S.P.A., were germinated at the H.S.P.A. Molokai Quarantine Station in 1962. Seeds from U.S. 62-1 produced four clones numbered from 1 to 4. Only clone No. 2 was included in this study. Crosses within U.S. 62-2 produced 100 plants numbered from 1 to 100. Four of these, numbered 62-2-20, 62-2-32, 62-2-34, and 62-2-73 were used in this study. All crosses between *Narenga* and *Sclerostachya* clones were made by Genetics Department, H.S.P.A., the hybrids were planted at the H.S.P.A. Kailua Field Laboratory in 1967.

TABLE I. PARENTAGE AND NUMBER OF HYBRIDS USED IN THE GENETICAL ANALYSIS

Cross No.	Type of Cross*	No. of hybrids
1	U.S. 62-2-20 x N. porphyrocoma	48
2	U.S. 62-1-2 x U.S. 62-2-34	48
3	N. porphyrocoma x U.S. 62-2-32	81
4	N. porphyrocoma x U.S. 62-2-73	93
5	U.S. 62-2-34 x U.S. 62-1-2	6
	Total	276

*U.S. 62-1 stands for Narenga, and U.S. 62-2 stands for Sclerostachya. U.S. 62-1 is a different plant from N. porphyrocoma mentioned in cross No. 1.

Peroxidase Isozyme Studies:

Electrophoresis runs were made under constant conditions. Fast runs were made at 300 volts, slow runs at 75 volts, using a Regulated Power Supply Heathkit Model IP-32. Leaf blade number 5 (counting from apex) was sampled, the whole leaf, or a representative sample if it was large, was homogenized and then mascerated. A small piece of filter paper (1 cm. x 2 mm.) was immersed in the macerated sample and then inserted into the gel which was prepared according to the method described by Smithies in 1955 improved by Poulik, 1957; Ashton and Braden, 1961; and Ashton and Lampkin, 1965.

A New Benzidine Iodide Staining for Peroxidase:

The conventional technique used for peroxidase staining after van Duijn as described by Siegel and Galston (1967) involving the use of benzidine as a hydrogen donor had many limitations. Contributions in its improvement have been suggested (Jermyn and Thomas, 1954; Owen, et al., 1958). In some cases other chemicals were substituted for benzidine, notably quaiacol, malachite green, o-ansidine, and o-diansidine. In this study benzidine was used as the dying substance. The method used was as follows:

1. 0.3 gm. of Benzidine were dissolved in 5 ml. acetic acid at 50° C, then 15 ml. distilled water were added to dilute the solution.

2. An equal volume of 3 percent hydrogen peroxide was added to the benzidine solution, when the color changed to faint blue, the mixture was poured over the gel in a glass container.

3. After the bright blue peroxidase bands appeared, the solution was decanted and the gel was washed twice in distilled water.

4. The gel was then placed in a very dilute aqueous solution of potassium iodide acidified with acetic acid. The exact concentration of the iodide was not determined, but a few crystals of the compound per 100 ml. of solution were quite satisfactory.

5. The iodide solution was poured off after 3 minutes, and the gel was washed and rinsed twice.

6. Before storing in refrigerator, small amounts of wash solution (10 parts methyl alcohol, 10 parts water, 2 parts glacial acetic acid and 1 part ethyl alcohol) were added for a short time to harden the gel. By this method the zymogram was preserved under refrigeration for several months.

One of the drawbacks of this technique was the critical requirement for definite speed in which every operation was performed. The gel should not be left in the benzidine peroxide mixture until the benzidine blue starts to become diffuse. The gel turns blue, due to the starch-iodine reaction, if iodine is used in high concentration or left in contact with the gel for a longer time than is needed to develop the zymogram. In order to remove the effect of over-staining, the gel was treated with 0.15N Sodium hydroxide until the blue color disappeared and the bands became clear. The gel background was restored to its previously clear condition and the bands changed into a dark brown stable color (Figure 1 with Figure 2).

The exact nature of the chemical reactions involved in this technique were not known. A few suggestions can be made. The

FIGURE 1. PEROXIDASE ISOZYME PATTERNS OF SIX HYBRIDS FROM THE PARENTAGE (U.S. 62-2-20 X N. PORPHYROCOMA), STAINED WITH THE BENZIDINE IODIDE METHOD.

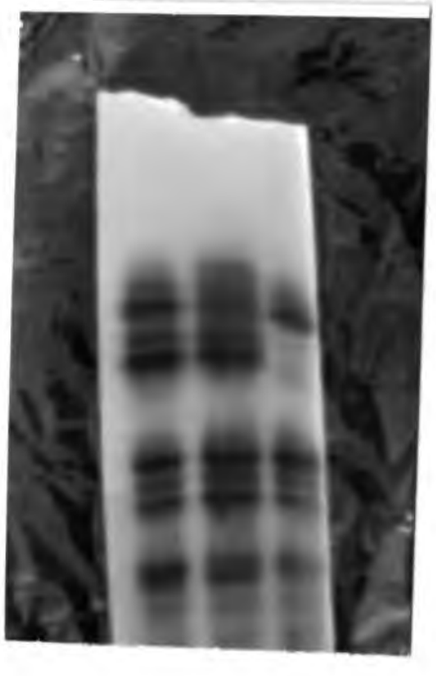

FIGURE 2. PEROXIDASE ISOZYME PATTERNS OF THREE HYBRIDS FROM THE PARENTAGE (U.S. 62-2-20 X N. PORPHYROCOMA), STAINED WITH THE BENZIDINE IODIDE METHOD AND TREATED WITH 0.15N SODIUM HYDROXIDE.

peroxidase enzyme oxidation reaction may be very simple and with simple compounds such as nitrite to nitrate and iodide to iodine (Chance, 1951). In some cases, the enzyme substrate has been shown to be more reactive than the enzyme itself (Chance, 1949). There was strong indication that the iodide was oxidized to iodine and the stable color was a direct result of the activated iodine action on the starch. The benzidine product in the enzyme substrate reaction might have reacted with the iodine and changed into a more stable form.

Isozyme Variations and Development:

Plant materials used in this study include the following clones of Narenga and Sclerostachya and their hybrids:

(1) U.S. 62-2-20

(2) U.S. 62-2-73

(3) U.S. 62-1-2

(4) N. porphyrocoma

(5) N. porphyrocoma x U.S. 62-2-32 Seedling No. 5

(6) N. porphyrocoma x U.S. 62-2-32 Seedling No. 20

(7) N. porphyrocoma x U.S. 62-2-73 Seedling No. 5

(8) N. porphyrocoma x U.S. 62-2-73 Seedling No. 6

(9) U.S. 62-2-73 x N. porphyrocoma Seedling No. 2

(10) U.S. 62-2-73 x N. porphyrocoma Seedling No. 3

(11) U.S. 62-2-20 x N. porphyrocoma Seedling No. 2

(12) U.S. 62-2-20 x N. porphyrocoma Seedling No. 29

(13) U.S. 62-2-34 x U.S. 62-1-2 Seedling No. 1

(14) U.S. 62-2-34 x U.S. 62-1-2 Seedling No. 5

(15) U.S. 62-1-2 x U.S. 62-2-34 Seedling No. 7

Callus tissues used in this study were derived from young inflorescences cultured under aseptic conditions. Coconut milk and 2,4-dichlorophenoxyacetic acid were used as the inducing agent for proliferation (Steward and Caplin, 1951). The culture media used throughout the study consisted of inorganic salt solution (Murashige and Skoog, 1962) with a thiamine and myo-inositol supplement as recommended by Linsmaier and Skoog (1965). Two percent sucrose and 10 gm. agar per liter were used. The inflorescences were cultured under 40-watt Sylvania Gro-lux Wide Spectrum lamps at a distance of approximately 27 cm. for one month. A representative sample of the callus produced from each inflorescence was transplanted into liquid media of three types:

(1) liquid media with coconut milk and 2,4-D (6 ppm.)

(2) liquid media with coconut milk and IAA (6 ppm.)

(3) liquid media with coconut milk only

Flasks containing the callus and the media were placed on a rotating shaker and after 15 days aliquot samples were used in electrophoretic runs after freezing and thawing followed by grinding in a mortar and pestle.

Staining for Leucineaminopeptidase (LAP) and Esterases was done according to the method described by Scandalios (1964). LAP was developed by incubating the starch gel for 45 minutes in a solution consisting of 40 mg. L-leucyl- B- naphthyl- amide- HCl, 50 mg. Black K

salt and 100 ml. tris-maleate Buffer. Esterases were demonstrated by incubating the starch gel for 45 minutes at 37° C. in 100 ml. of phosphate buffer containing α-naphthyl acetate and fast blue RR salt as a dye coupler.

RESULTS AND DISCUSSION

Isozyme Patterns of Peroxidases:

In all the hybrids, marked heterogeneity was observed. This was expected since the materials dealt with were the F_1 hybrids between two genera with complex polyploidy constitutions. Sound genetic analysis for some of the isozyme combinations appeared to be very difficult if not impossible. Four groups of isozyme patterns were observed, a cathodic group plus three anodic groups based on their rate of migration towards the anode. These groups are illustrated in Figs. 3, 4, 5, 6 and 7. The four groups were quite variable within themselves and they were present in any combination with the other forms, indicating considerable complexity in their inheritance. These four groups were quite persistent and in no instance was a marked change observed.

In general, about 13 bands of peroxidase isozymes appeared in the anodic region and not more than 6 bands appeared in the cathodic region. Bands in the cathodic region were not always present and were missing from leaf tissue in three of the parents, U.S. 62-2-73, N. porphyrocoma, and U.S. 62-1-2. The isozyme patterns of the parents are shown in Figs. 3 and 4. Other hybrid patterns are also shown in Figs. 5, 6, 7, 8, 9, and in Table II.

The isozymes were divided into four major zones (Table II and Fig. 3) according to their rate of migration. The first is a cathodic group which, at most, produced six bands and in the majority of cases fewer bands were produced by leaf tissue, although they were observed in zymograms from inflorescence tissue. This group of isozymes was not present in leaf tissue in each plant. On staining a dense stain always

visible in the sample filter papers, indicated the presence of an electrically neutral peroxidases or peroxidases with larger molecules. Since the genetic nature of this enzyme could not be studied and there was a possibility of the presence of some kind of a polymer which might obscure the results, these bands were not included in the genetic analysis. To add to the complexity it was noticed that in some crosses where neither of the parents showed these bands, the offspring showed some of them. The possibility of a hybrid enzyme is quite obvious. Similar results were observed in maize by Beckman, et al. (1964).

The second group constitutes the nearest group to the origin in the anodic region. It was given the designation (A) and it is divided into three parts. The two parts nearest the origin A_1, and A_2 were not included in the analysis due to the lack of distinct differences in the bands observed. Sub-group A_3 was by far the most interesting and showed clear-cut mendelian ratios. In the A_3 position either one or two bands were present. A single band indicated either a homozygote or a heterozygote with a null allele (Burns and Johnson, 1967). In this respect, it is postulated that multiple alleles are most likely predominant in this kind of material. Table III shows the number of individuals observed in each class for sub-group A_3 together with their respective Chi-square values. The two Narenga clones were believed to be homozygous for the A_3 position since they showed only one band and their genetic constitution may be as follows:

Perox. $A_3(1)$/Perox. $A_3(1)$

With the exception of U.S. 62-2-32, all other members of the

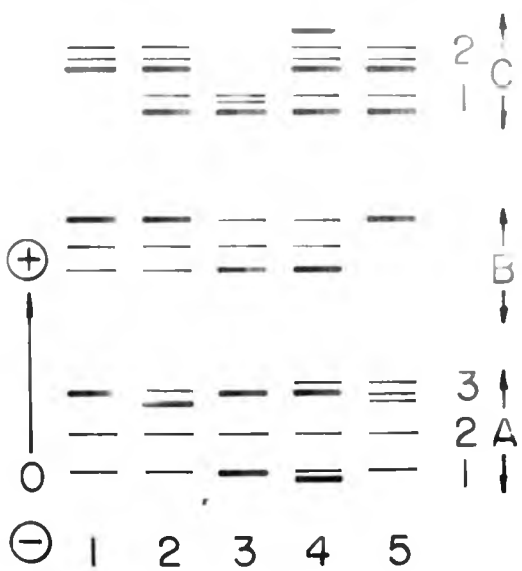

FIGURE 3. A DIAGRAMMATIC REPRESENTATION OF THE FIVE PARENTS AS FOLLOWS: 1) N. PORPHYROCOMA, 2) U.S. 62-2-20, 3) U.S. 62-2-32, 4) U.S. 62-2-34, AND 5) U.S. 62-2-73 SHOWING THE PEROXIDASE GROUPS A, B, C. U.S. 62-1-2 WAS SIMILAR TO N. PORPHYROCOMA. THE "O" INDICATES THE ORIGIN.

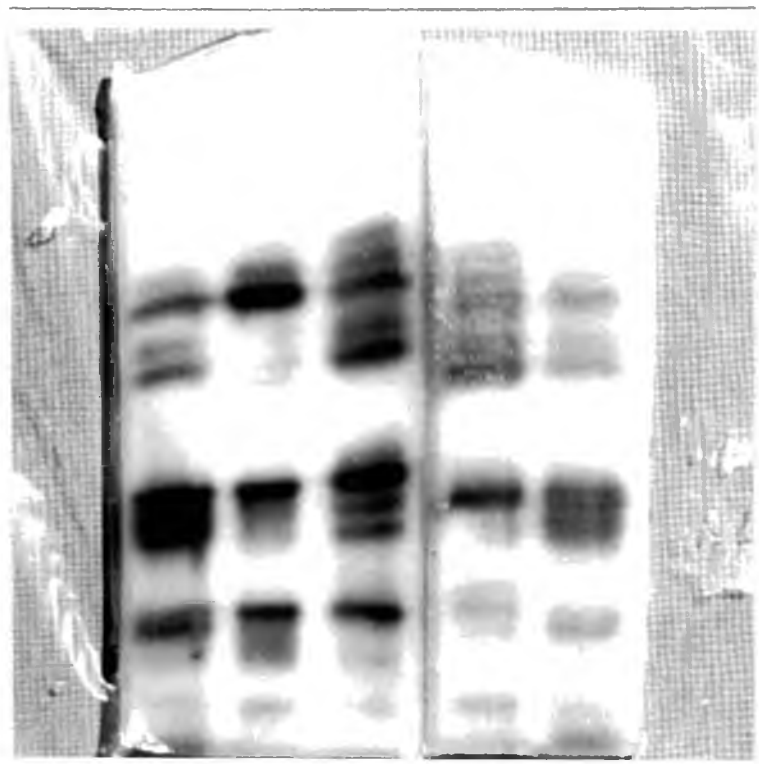

FIGURE 4. A PHOTOGRAPH OF THE GEL SHOWING THE PEROXIDASE ISOZYME PATTERNS OF THE PARENTS ARRANGED FROM LEFT TO RIGHT AS FOLLOWS: 1) U.S. 62-2-20, 2) N. PORPHYROCOMA, 3) U.S. 62-2-34, 4) U.S. 62-2-73, AND 5) U.S. 62-2-20. THE ARROW INDICATES THE ORIGIN.

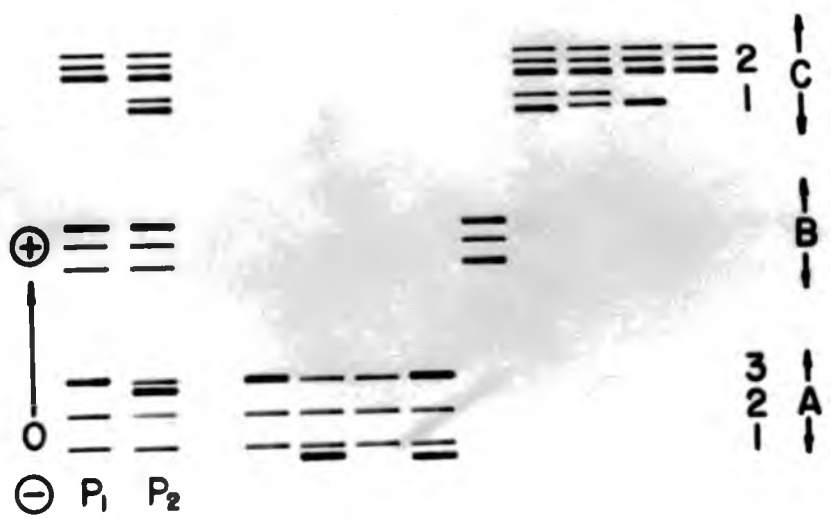

FIGURE 5. A DIAGRAMMATIC REPRESENTATION OF THE PEROXIDASE ISOZYME PATTERNS REVEALED IN THE CROSS U.S. 62-2-20 X N. PORPHYROCOMA. (P_1 STANDS FOR N. PORPHYROCOMA AND P_2 STANDS FOR U.S. 62-2-20.)

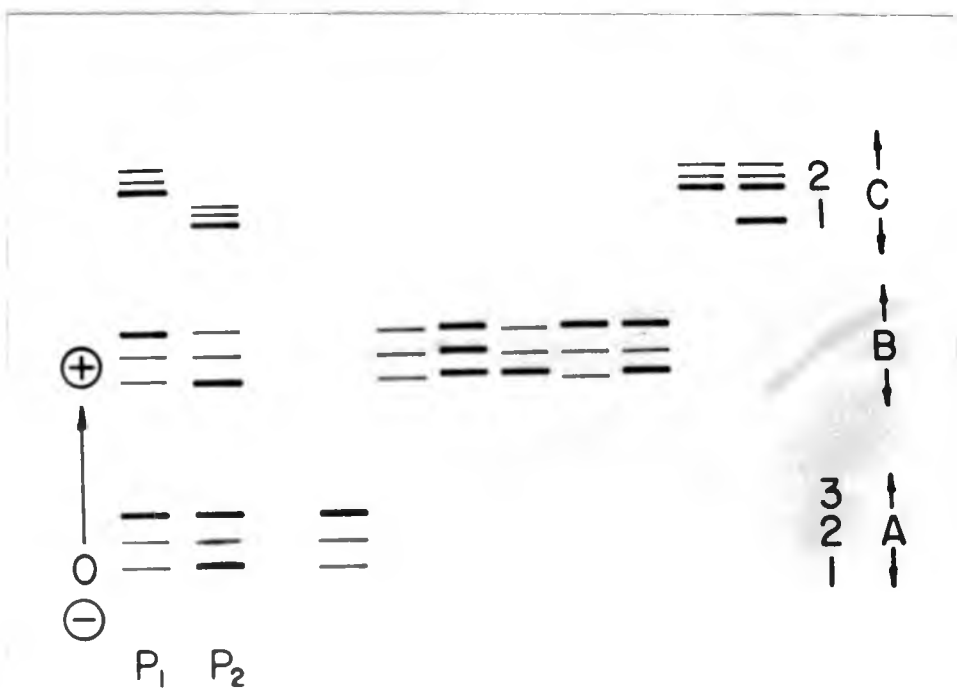

FIGURE 6. A DIAGRAMMATIC REPRESENTATION OF THE PEROXIDASE ISOZYME PATTERNS REVEALED IN THE CROSS N. PORPHYROCOMA X U.S. 62-2-32. (P_1 STANDS FOR N. PORPHYROCOMA AND P_2 STANDS FOR U.S. 62-2-32.)

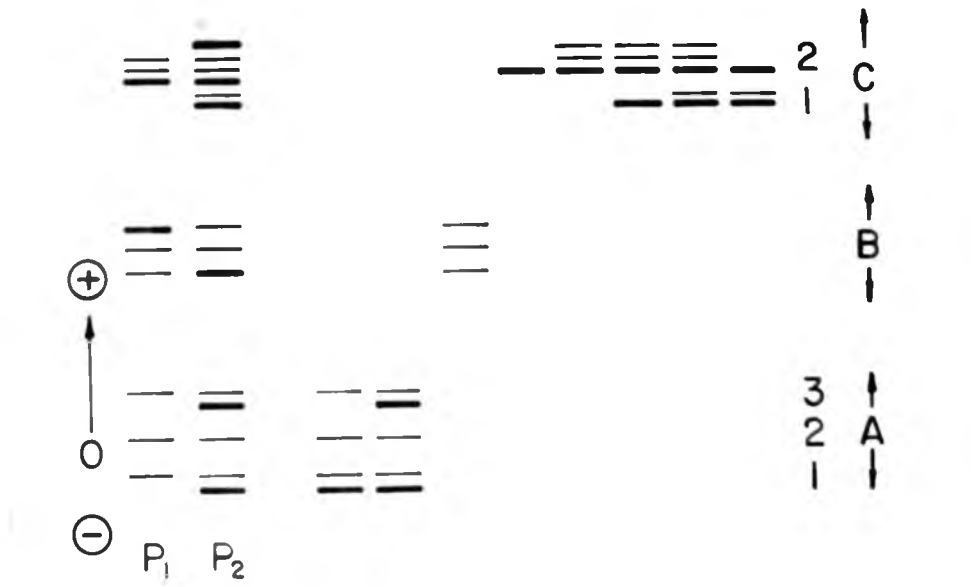

FIGURE 7. A DIAGRAMMATIC REPRESENTATION OF THE PEROXIDASE ISOZYME PATTERNS IN THE CROSS U.S. 62-1-2 X U.S. 62-2-34. (P_1 STANDS FOR U.S. 62-1-2 AND P_2 STANDS FOR U.S. 62-2-34.)

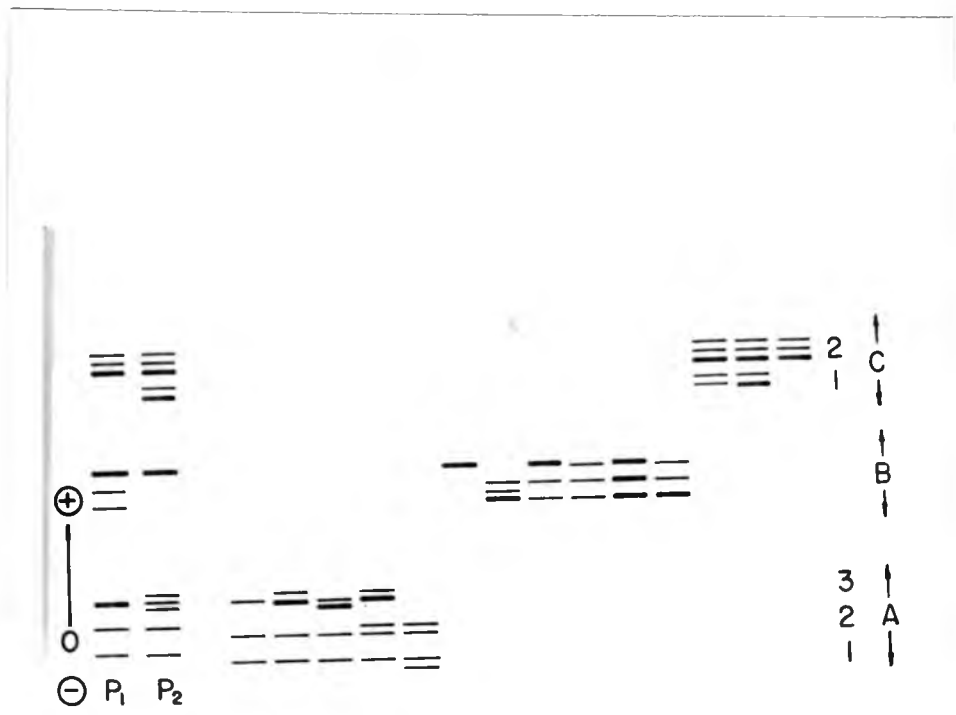

FIGURE 8. A DIAGRAMMATIC REPRESENTATION OF THE PEROXIDASE ISOZYME PATTERNS IN THE CROSS N. PORPHYROCOMA X U.S. 62-2-73. (P_1 STANDS FOR N. PORPHYROCOMA AND P_2 STANDS FOR U.S. 62-2-73.)

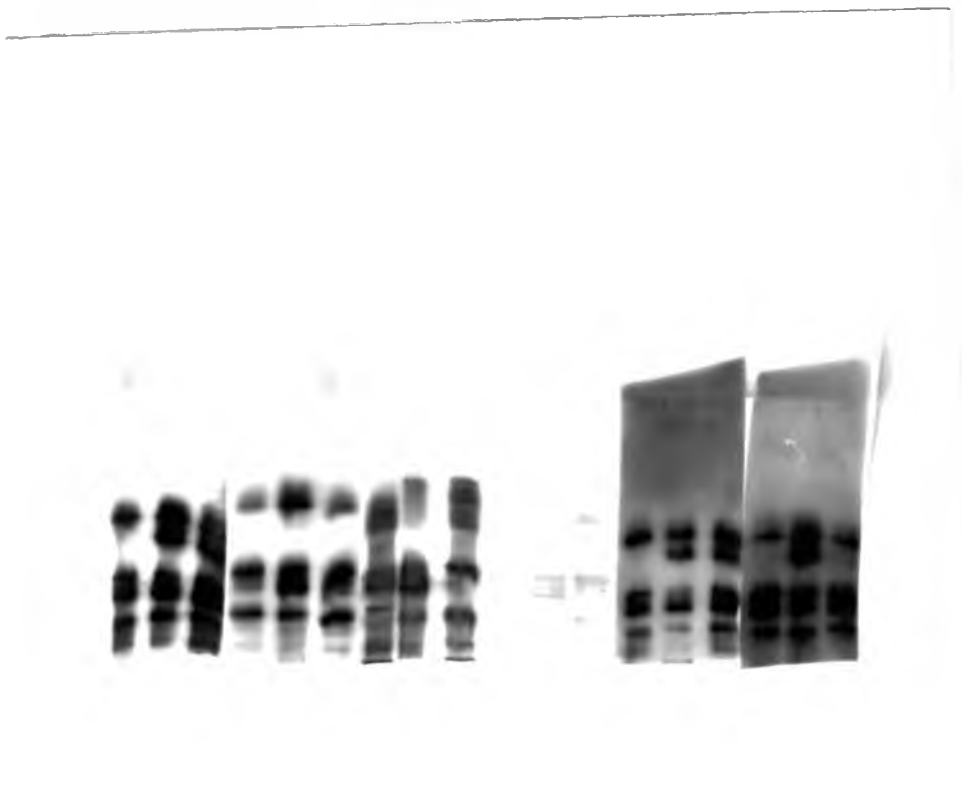

FIGURE 9. A PHOTOGRAPH OF PEROXIDASE ISOZYME PATTERNS FROM 18 SAMPLES IN THE CROSS U.S. 62-2-20 X N. PORPHYROCOMA UNDER FAST RUNS. THE FIGURE ILLUSTRATES THE VARIABILITY SHOWN IN THE ANODIC PORTION OF THE GEL.

TABLE II. A SUMMARY TABLE OF FIGURES FIVE TO EIGHT

Peroxidase Sub-group		Cross No.* 1	2	3	4
		Number of hybrids showing bands			
A_1	1 band	22	54	81	66
	2 bands	26	0	0	14
A_2	1 band	48	54	80	52
	2 bands	0	0	0	28
A_3	1 band	25	29	81	11
	2 bands	23	25	0	69
B	1 band	0	0	0	**
	3 bands	48	54	81	
C_1	None	13	6	38	38
	1 band	20	26	43	12
	2 bands	15	20	0	40
C_2	1 band	0	10	0	0
	3 bands	48	44	81	90

*Cross Nos. 1, 2, 3, and 4 are U.S. 62-2-20 x N. porphyrocoma, U.S. 62-1-2 x U.S. 62-2-34, N. porphyrocoma x U.S. 62-2-32, N. porphyrocoma x U.S. 62-2-73, respectively. The figures in the table indicate the numbers of clones showing each type.

**This cross gave a very confusing number of bands and is not included.

Sclerostachya clones studied were apparently heterozygotes with a number of multiple alleles. In U.S. 62-2-20 and U.S. 62-2-34, two bands were observed; their genetic constitution may be as follows;

Perox. $A_3(1)$/perox. $A_3(2)$

U.S. 62-2-73 showed three bands in the A_3 position, since the enzyme may be a dimer, it can still be considered as a heterozygote with a different multiple allele constitution (Burns and Johnson, 1967). Among the F_1 progeny of U.S. 62-2-73, 69 clones with two bands and 11 clones with one band were observed. There were none with three bands. Other genes may be involved.

In cross No. 3 (N. porphyrocoma x U.S. 62-2-32), all the clones showed a single band indicating that both parents were homozygous at this locus or at least U.S. 62-2-32 was heterozygous with a null allele, i.e. an allele which is nonfunctioning (Table III).

The peroxidase B group was the second fastest and its position in the gel was constant. The other crosses produced similar patterns in the position and number of bands. Three bands appeared either heavily or lightly stained in the various progenies and not all in the same order. U.S. 62-2-73 and some of its hybrids showed only a single band. Table IV shows the number of progenies studied and the number of bands observed in each cross. In group B the isozyme was regarded as a dimer and two pairs of genes were involved, presumably in a homozygous condition. However, the presence of multiple alleles cannot be disregarded and must be considered as quite possible. No analysis was made since all the progenies from each cross with the exception of cross

TABLE III. A SUMMARY TABLE FOR THE PEROXIDASE A_3 SUBGROUP WITH THEIR CHI-SQUARE VALUES AND PROBABILITIES

Cross No.	Type of Cross	No. of Bands observed* 1	2	No. of progenies examined	Chi-square for 1 : 1 ratio	P
1	U.S. 62-2-20 x *N. porphyrocoma*	25	23	48	0.082	.90-.75
2	U.S. 62-1-2 x U.S. 62-2-34	29	25	54	0.296	.75-.50
3	*N. porphyrocoma* x U.S. 62-2-32	81	0	81		
4	*N. porphyrocoma* x U.S. 62-2-73	11	69	80		

*This includes both heavily and/or lightly stained bands.

TABLE IV. A SUMMARY TABLE FOR PEROXIDASE B GROUP IN CROSS NOS. 1, 2, 3, 4

Cross No.	Type of Cross	No. of Bands observed* 3	1	No. of progenies examined
1	U.S. 62-2-20 x *N. porphyrocoma*	48	0	48
2	U.S. 62-1-2 x U.S. 62-2-34	54	0	54
3	*N. porphyrocoma* x U.S. 62-2-32	81	0	81
4	*N. porphyrocoma* x U.S. 62-2-73	44	19	63**

*This includes both heavily and/or lightly stained bands.

**Twenty-three of the 86 hybrids examined are not included here due to difficulty in determining number of bands.

No. 4 showed the three bands.

In U.S. 62-2-73, very interesting results were obtained in the B group. Of the 86 clones studied, 44 clones had 3 bands, 19 clones showed a single band and 23 clones had 3 bands with 2 of the three very faint and narrowly spaced. This was a unique pattern not previously observed. This new form may be a hybrid enzyme.

The fourth group, peroxidase C, was the fastest migrating in plant materials studied. It was more complex than the B group, since it contained two different sub-groups which appeared to be inherited separately. These sub-groups were given the designation C_1 and C_2 according to their respective positions from the origin. Both sub-groups were made up of at least one heavy band that stained heavily and one or more lightly stained bands. Although the heavy bands were frequently associated with the faint bands, there was strong indication that they were inherited separately. In two crosses (Nos. 2 and 3), the hybrids showed a single heavy band in either peroxidase C_1 or C_2 positions. In this study only these two heavy bands were analyzed genetically (Tables V and VI).

In cross No. 1 (U.S. 62-2-20 x N. porphyrocoma), 22 progenies produced a heavy band, 26 did not. The presence or absence of the heavy band is most likely a 1 : 1 ratio (Chi-square = .332 P = .75-.50). Since C_1 was not detected in N. porphyrocoma, U.S. 62-2-20 may be heterozygous for this locus with a null allele; and its genetic constitution for the heavy band locus is presented as follows:

Perox. $C_1(1)$/Perox. $C_1(0)$

TABLE V. A SUMMARY TABLE FOR PEROXIDASE C_1 SUBGROUP, WITH CHI-SQUARE FOR 1 : 1 RATIO

Cross No.	Type of Cross	No. of Bands observed* 1	None	No. of progenies examined	Chi-square for 1 : 1 ratio	P
1	U.S. 62-2-20 x *N. porphyrocoma*	22	26	48	.332	.75-.50
2	U.S. 62-1-2 x U.S. 62-2-34	26	22	48	.332	.75-.50
3	*N. porphyrocoma* x U.S. 62-2-32	43	38	81	.310	.75-.50
4	*N. porphyrocoma* x U.S. 62-2-73	52	38	90	2.16	.25-.10

*The figures represent the number of hybrids showing presence or absence of the C_1 heavy band.

In cross No. 2, U.S. 62-1-2 x U.S. 62-2-34, 26 out of 48 clones investigated showed the presence of a heavy band in the sub-group C_1 (Table V).

In cross No. 3, involving 62-2-32, 43 clones showed the presence of a single heavy band in the C_1 position and 38 showed no band at all. The genetic constitution of U.S. 62-2-32 for C_1 sub-group was also heterozygous and it can be written as follows:

Perox. $C_1(2)$/Perox. $C_1(0)$

A different notation from U.S. 62-2-20 was used because of differences found in the minor bands.

Cross No. 2 (N. porphyrocoma x U.S. 62-2-73) showed the following: 52 clones with a heavy band, 38 clones with either a light band or none at all. Results obtained were obscure due to lack of distinct differences in the bands observed.

The peroxidase sub-group C_2 was the fastest group in this material and appears to have simplest inheritance, Table VI. It consisted, in the majority of cases studied, of one major band associated with a variable number of minor ones. In cross No. 2, this major band appeared alone indicating its independence. In cross No. 3 where one of the parents, U.S. 62-2-32, did not have this band, it was present in all the hybrids. This was indication for the homozygosity of N. porphyrocoma at this locus. In the other crosses, this band was present and it was associated with a variable number of faint bands. In the cross No. 2, U.S. 62-1-2 x U.S. 62-2-34 (Table VI), the C_2 was heavy band by itself in ten of the 54 progenies.

TABLE VI. A SUMMARY TABLE FOR PEROXIDASE C_2 SUBGROUP

Cross No.	Type of Cross	No. of bands observed* 1	3	No. of progenies examined
1	U.S. 62-2-20 x *N. porphyrocoma*	0	48	48
2	U.S. 62-1-2 x U.S. 62-2-34	10	44	54
3	*N. porphyrocoma* x U.S. 62-2-32	0	81	81
4	*N. porphyrocoma* x U.S. 62-2-73	0	90	90

*The figures represent the number of clones showing each type. Each clone with three bands includes the heavy C_2 band also.

The validity of dividing the peroxidase systems, in the plants studied, into groups and sub-groups as pointed out previously, awaits verification. A possible explanation for the simple genetic ratios presented in this enzyme system in a population of hybrids claimed by some people to be of a complex polyploid nature, can be found in the enzyme system themselves. Possibly the peroxidase systems in these two genera were the outcome of intensive evolutionary processes in which major genes for this enzyme were centered in a few chromosomes in one or two basic sets. Regular meiosis in the two genera was reported (Raghavan, 1951), and accordingly the genetic behavior of the two genera might prove to be simple and not complex as originally anticipated. However, unpublished information at the H.S.P.A. and the Department of Agronomy and Soil Science, University of Hawaii, indicate that the clone N. porphyrocoma is nearly pollen sterile, with completely irregular meiosis. This may have considerable influence on the inheritance of the peroxidase isozyme systems.

Slow Electrophoretic Runs:

A heavy band was found in each of the peroxidase sub-groups, C_1 and C_2. The heavy bands were separated from the light bands since each heavy band was present or absent independently of the light bands. The heavy bands usually appearing with some minor bands varying in number in both sub-groups C_1 and C_2, independently appeared in the progenies of some of the crosses. The presence or absence of each of these two bands was considered as genetically controlled and genetic analysis was made accordingly.

Investigations to find other clues in support of the above trend were made. In crosses Nos. 1 and 2, samples from each of 18 progenies were subjected to slow electrophonetic runs. The voltage was reduced to 75 instead of 300 volts and the time adjusted so that the borate front reached a distance equal to that produced by 300 v. in a 3 hour period.

The zymogram was stained to show the magnitude and positions of the bands. The same general isozyme patterns were observed; however, in the peroxidase groups B and C, the bands had a marked tendency to be closer together and to form broad and ill defined units. Most of them could still be identified (Fig. 10). The division of isozyme patterns in the three major groups is most likely a correct one since results observed for the 75 volt runs were similar to those obtained from 300 volt runs.

To investigate the within group sub-divisions, particularly the isolation of the two heavy bands in peroxidase sub-groups C_1 and C_2, two points must be investigated. The first point is the possibility that the isozymes in these sub-groups are dimers or tetramers. That they are not was indicated by the presence of the heavy band in both sub-groups independently and unassociated with the other minor bands. This was seen in two crosses involving the genus Narenga with the Sclerostachya clones, U.S. 62-2-34 and U.S. 62-2-32. In the first cross, among all the progenies studied, which had the C_1 sub-group, only the heavy band was observed. In the second cross, the heavy band in sub-group C_2 was observed in some of the progenies studied.

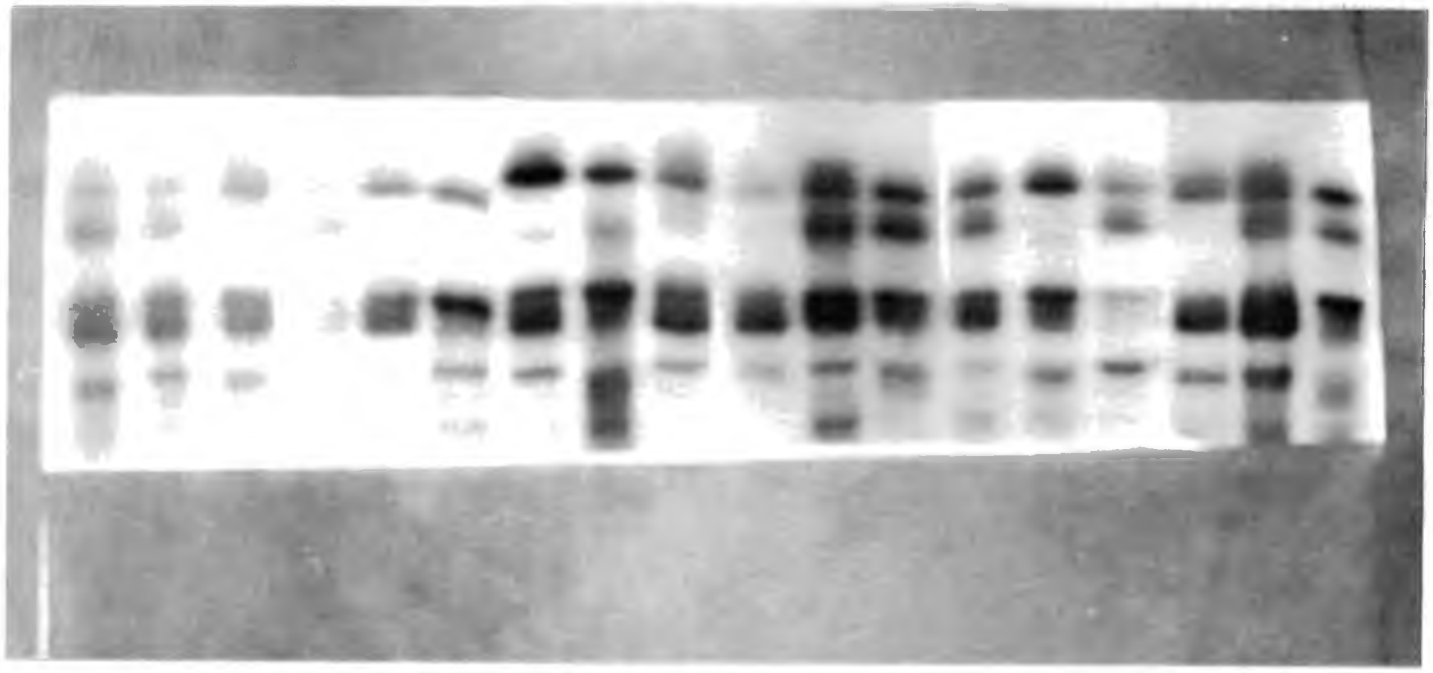

FIGURE 10. A PHOTOGRAPH OF ISOZYME PATTERNS IN 18 SAMPLES OF THE CROSS U.S. 62-1-2 X U.S. 62-2-34 UNDER SLOW ELECTROPHORETIC RUNS. THE ANODIC PORTION OF THE ZYMOGRAM IS REPRESENTED.

The other point investigated was based on the theory of moving boundary electrophoresis of a reversibly interacting system, presented by Cann and Goad (1965) which may be summarized as follows: A single macro-molecule will react with the uncharged molecule of the solvent, and it was expected that if the interaction is highly cooperative the system will behave like a mixture of two stable non-interacting macro-molecules.

$$P + nHA \qquad P(HA)n$$

where P = protein and P(HA)n = its complex formed by binding of n moles of a small uncharged constituent of the solvent HA. In this case, such proteins could easily be misinterpreted as indicating true heterogeneity. To eliminate this possibility, samples from different crosses were run and finally the gel was sliced according to the method described by Markert and Moller (1959). One of the slices was stained and then the two gels were superimposed on each other with clear plastic in between. The two bands (C_1 and C_2 heavy bands) under investigation were removed from the nonstained gel and immediately run in a new electrophoretic setup. After staining the isozymes revealed themselves as single bands in comparable positions. Accordingly, no fractional material was involved in the system investigated.

The outcome of this work emphasized the fact that when these two bands appeared separately from their groups, they were separately inherited. The nature of the genes involved in the production of these bands was obscure, and they could very likely be structural or regulating in nature.

Isozyme Variations and Development:

Urata and Rotar (unpublished) were able to produce intact plants from callus tissue originated from young inflorescences in some species of the family Gramineae. Their technique was applied to some members of the genus Saccharum (Talballa, et al., unpublished). Callus tissues grown in a media with 2,4-D (2,4-dichlorophenoxyacetic acid) produced different isozyme patterns when compared with callus tissue grown in a medium lacking 2,4-D. Since the callus was always derived from similar plant tissues, it was suggested that these isozymes variations were due to developmental change. Nevertheless, the possibility that these changes are specific for certain plants must be kept in mind.

The original callus produced directly in the agar media was very heterogenous for each inflorescence. Patches of white callus together with small green and red specks occurred. Except for the red specks, similar types of callus tissues were previously obtained from some members of the genus Saccharum.

Isozyme Patterns of Leaves, Inflorescences and Callus Tissues:

Callus tissues, derived from young inflorescence samples grown in three liquid media, (a) with 2,4-D (2,4-dichlorophenoxyacetic acid); (b) with IAA, 3-indoleacetic acid; and (c) coconut milk alone; were compared with leaf and young and old inflorescence tissue. Young inflorescences were in the late boot stage. Whole young inflorescences were used to insure the inclusion of all its tissues in the sample. In the old inflorescences, representative samples were taken. Three enzyme systems were studied; esterase, L-amino-peptidase and peroxidase.

a) Esterase in leaf, inflorescences and callus tissues:

No esterases were observed from young inflorescences of all the clones studied. Esterases from leaves showed a main slow and broad band or groups of bands which were not distinctly resolved. Esterases from callus tissue derived from all the clones investigated, produced a complex fast group of bands. All clones showed the same pattern.

Two points in these findings are worth special consideration: first, the complete absence of the esterase system in inflorescences; secondly, the shift from slow migrating bands found in the leaves to a fast and a complex system in callus tissues.

b) L-amino-peptidase in leaf, inflorescence and callus tissues:

L-amino-peptidase enzyme patterns were similar to the esterases. There was complete absence of this system in young inflorescence. The leaf showed a slow migrating ill-defined group of bands. A fast migrating group was found in callus tissue. This was broad and could not be distinctly resolved. It was interesting to note that no L-amino-peptidase was found in very young leaves, which were still enclosed in the sheaths of the older leaves.

c) Peroxidase in leaf, inflorescence and callus tissues:

Again, in this complex enzymatic system, basic differences among the three tissues were found. This system can be divided into two parts; an anodic system and cathodic

system. The leaf in general has a very complex anodic system, which in some cases approached 15 bands. The cathodic portion of the peroxidase system was very reduced in both old and young leaves; in a few clones where it was detected, it was faint and did not exceed one or two bands in number (Fig. 11).

Old inflorescences, which have already emerged and turned purplish in color, showed a complex system in the anodic portion analogous to that found in the leaf. Young inflorescences showed a gradual simplification of the anodic system extending from the farthest group from the origin to the slowest migrating bands. In all cases the peroxidase A group was always present. In the cathodic part there was a more complex system than that found in the leaves. In some leaves the cathodic portion was completely absent, in the inflorescence it was always present. Up to 6 bands were observed in the cathodic portion for inflorescences.

Young inflorescences tended to show a more complex system in the cathodic region than older ones. A general feature of the inflorescence was the continuous diffusion in the anodic system. All the bands merged into one another in a continuous shadow-like trail. This was also noticed in callus tissue. It was probably caused by a general degradation or breaking down of the enzyme system.

In the callus tissues investigated, the cathodic region had an elaborate, complex system. The intensity of bands as well as their number was greater than those in the leaf and

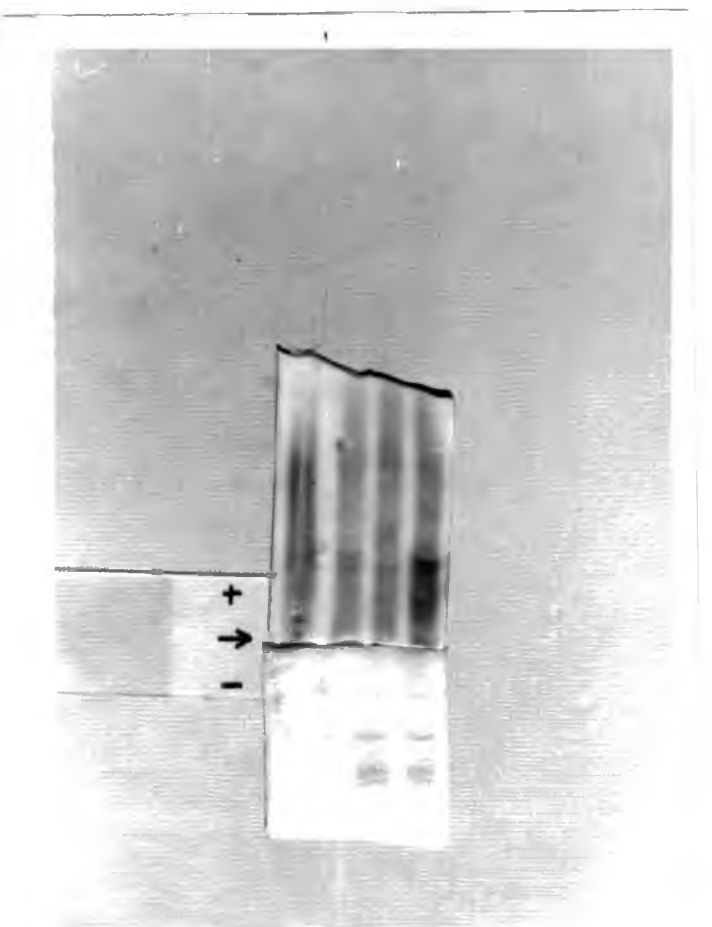

FIGURE 11. PEROXIDASE ISOZYME PATTERNS IN THE ANODIC AND CATHODIC REGIONS OF THE GEL SHOWING THE TYPE OF ISOZYME DIFFUSION FROM CALLUS TISSUES OF FOUR DIFFERENT CLONES. ALSO NOTICE THE VERY WELL DEVELOPED CATHODIC PORTION.

inflorescences. There were several points in common between the callus tissue and the tissues of the young inflorescences. The fastest migrating bands in the anodic region were very much reduced in intensity. Although the number of bands in the peroxidase C_2 sub-group did not seem to be affected as they were always very faint and took a longer time to develop in the stain. There was the marked phenomenon of diffusion as if the bands were linked by a continuous system of undefined isozymes (Fig. 11).

d) Isozyme patterns in callus tissue grown on different media:

Isozyme patterns of the callus tissues grown in three different culture media were compared for esterase, L-amino-peptidase and peroxidase. The same general pattern was found for each culture media. As mentioned before, a major shift in production of esterase and L-amino-peptidase was found in callus tissue in comparison to the leaves. In both LAP and esterase systems a change towards a fast migrating group was found. By far the most conspicuous change is in the esterase enzyme system, where a number of well defined bands were found. All the clones investigated showed fewer bands in this system when grown as callus in a media with the inducing hormones, 2,4-dichlorophenoxyacetic acid and indoleacetic acid (Figs. 12 and 13).

In the LAP system, variation occurred only in two clones, N. porphyrocoma clone and Sclerostachya clone No. U.S. 62-2-73 (Fig. 14). No well defined bands were produced by this enzyme

FIGURE 12. ESTERASE ISOZYME PATTERNS IN CLONE NO. 2 FROM THE CROSS N. PORPHYROCOMA X U.S. 62-2-73. FROM LEFT TO RIGHT: 1) CALLUS TISSUE FROM A MEDIUM WITH COCONUT MILK: 2) CALLUS TISSUE FROM A MEDIUM WITH 2,4-D; 3) CALLUS TISSUE FROM A MEDIUM WITH COCONUT MILK. THE ARROW INDICATES THE ORIGIN.

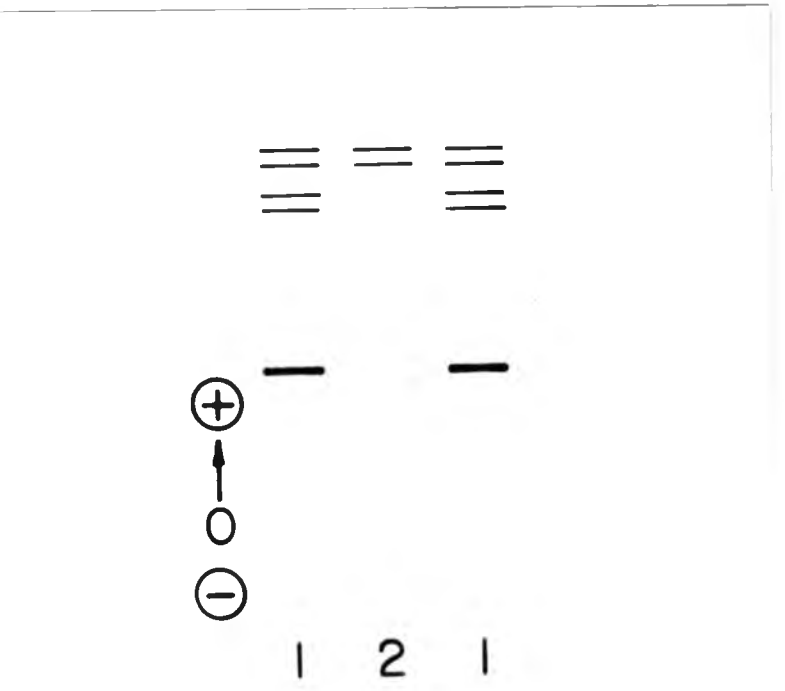

FIGURE 13. A DIAGRAMMATIC REPRESENTATION OF ESTERASE ISOZYME PATTERNS IN CLONE NO. 2 FROM THE CROSS U.S. 62-2-73 X N. PORPHYROCOMA. FROM LEFT TO RIGHT: 1) CALLUS TISSUE FROM MEDIUM WITH COCONUT MILK; 2) CALLUS FROM A MEDIUM WITH 2,4-D; 3) CALLUS TISSUE FROM A MEDIUM WITH COCONUT MILK. THE (0) INDICATES THE ORIGIN.

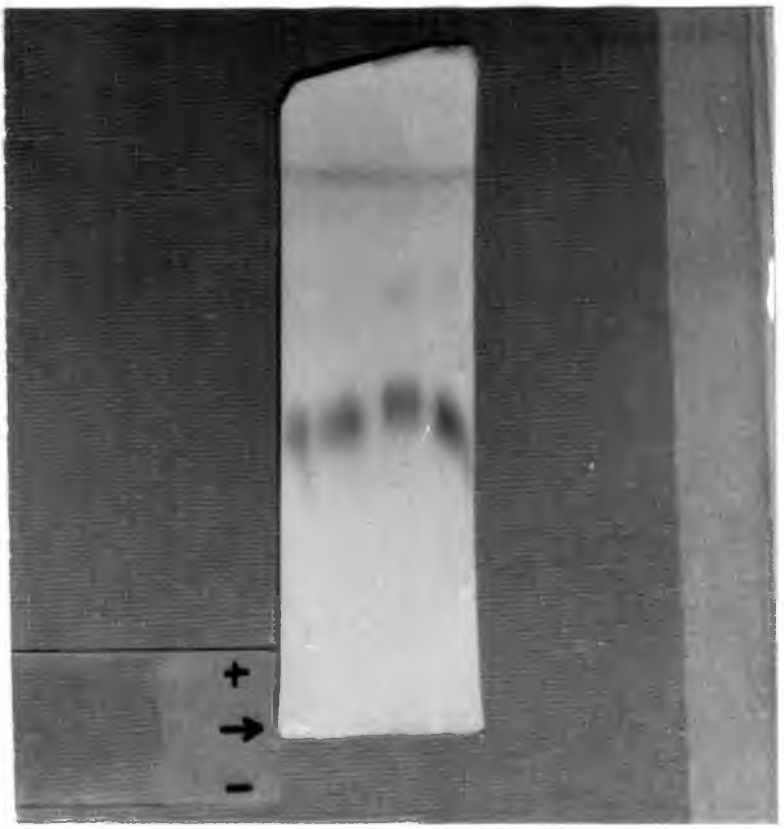

FIGURE 14. LAP ISOZYME PATTERNS IN FOUR SAMPLES FROM U.S. 62-2-73. FROM LEFT TO RIGHT: 1) CALLUS TISSUE FROM MEDIA WITH 2,4-D; 2) AND 3) CALLUS TISSUE FROM MEDIA WITH COCONUT MILK; AND 4) CALLUS TISSUE FROM MEDIA WITH IAA. THE ARROW INDICATES THE ORIGIN.

system. S. fusca U.S. 62-2-73 and N. porphyrocoma produced different patterns. A conspicuously broader LAP band was found in callus grown on coconut media above as compared to that grown in a media containing coconut milk plus 2,4-D or IAA.

In the peroxidase system, all the clones showed similar patterns with a very well developed cathodic portion and a greatly reduced anodic system. Clone N. porphyrocoma showed some differences in peroxidase systems between callus derived from media with coconut milk and that which had a hormonal supplement. A more complex cathodic system was found in media lacking an inducing hormone.

e) Formation of plants:

Plant development from callus was not observed for 2 months even after two transfers. After this period of time roots were formed, in most cases, deformed leaves, and bracts were observed. A more organized form of growth was observed after three transfers. When callus tissue was transferred to a solid media, with coconut milk as described previously, it turned into a distinct white mass with some green spots unevenly distributed throughout the tissue. These green spots increased in number and in size, and in so doing, the rate of growth in the nongreen portion was checked. Bracts and leaf-sheath-like structures were formed from these green areas. The leaf-sheath-like structures elongated considerably and a succession of leaf production followed. Roots tended to be

formed at this stage although independent roots were formed at a somewhat earlier stage, largely from the nongreen part of the callus tissue. These independent roots died after a short time.

After the plants reached a certain stage in development with a number of leaves they tended to form roots. The plants survived and continued to grow for a long time; the only limiting factor for continuous growth was the subsequent exhaustion of the nutritive medium. Callus tissue in association with these plants tended to survive for a very long time and apparently in a very healthy condition. Plants were obtained from callus tissue of several hybrids as well as from U.S. 62-2-73 (Fig. 15).

FIGURE 15. A PHOTOGRAPH ILLUSTRATING: 1) CALLUS TISSUE; 2) EARLY FORMATION OF SHOOTS; 3) COMPLETE PLANTS, FROM CLONE NO. 7 IN THE CROSS U.S. 62-1-2 X U.S. 62-2-34.

CONCLUSIONS

Peroxidase Isozyme Patterns:

In a study such as this, one needs to have both F_1 and S_1 progenies from each parent in order to reach sound conclusions. The plant materials used were only F_1 progenies and they were not in adequate numbers. The analysis should be studied with these limitations in mind.

The peroxidase isozyme patterns in the two genera, Narenga and Sclerostachya were comparable in many respects. In the anodic portion of the gel similar groups with respect to their position and their number of bands were found in all the parents and their hybrids.

All the clones representative of Sclerostachya fusca were comparable with the exception of the clone U.S. 62-2-73. This clone is slightly different from the others and its genetic analysis gave ambiguous ratios. U.S. 62-2-34 was different in a number of aspects. It showed a heavy band at the extreme end of the peroxidase C_2 sub-group, a band not detected in the other clones in the genus Sclerostachya. In cross No. 2 (U.S. 62-1-2 x U.S. 62-2-34) 10 hybrid plants showed only one band in peroxidase C_2 sub-group. Again this was not found in any of the other crosses. Since the clone U.S. 62-1-2 was the one used in this cross and not N. porphyrocoma which was used in the other crosses, this difference could be due to either one of the two parents. As far as the genus Narenga is concerned one point should be presented. The clone N. porphyrocoma indicated a high degree of homozygosity since certain patterns found only in this clone were shown

in all the hybrids investigated in the cross N. porphyrocoma x U.S. 62-2-32.

Isozyme Variation and Development:

Isozyme patterns for three enzyme systems; esterase, L-amino-peptidase and peroxidase where compared in the leaves, young inflorescences and callus tissue. A more complex system was present in the leaf especially in the peroxidase anodic groups.

The cathodic portion of the peroxidase system was very complex in callus tissue. This portion of the enzyme might have something to do with active cell division and growth. In connection with this idea further studies using growth stimulants or growth retardants might clarify this point.

The callus tissue showed a fast migrating complex system in the esterase and L-amino-peptidase isozymes. The inflorescences and the leaves showed a slow migrating system. Accordingly the esterase and the L-amino-peptidase systems could be resolved into different portions associated with different stages of growth, in a similar manner to the peroxidase system.

Plant Formation:

Three different forms of callus tissue were found, red, white and green. The white portion gave mainly roots, and the green portion gave bracts, leaves and plants.

SUMMARY

Peroxidase patterns were studied electrophoretically in clones of the genera Narenga and Sclerostachya and in some of their hybrids. During this study a new technique for staining the peroxidase zymogram was developed. This staining method utilizes two staining substances simultaneously, namely benzidine and potassium iodide, leading to a better manifestation and preservation of the isozyme bands in the zymogram. This new method was described, and some relevant information about its chemical nature was presented, together with some of its drawbacks.

The peroxidase isozymes in mature leaf blades were divided into groups and sub-groups according to their position in the gel. Marked similarities were found in the two genera and in their hybrids according to the position and the number of bands in these groups. Three of these groups lying in the anodic portion of the gel were analyzed genetically. In two groups simple Mendelian ratios of 1:1 were found. To investigate the validity of the division of the peroxidase isozymes into groups and sub-groups some work involving slow electrophoretic runs was done. In addition, a test of the hypothesis of moving boundary electrophoresis of reversibly interacting systems, as presented by Cann and Goad in 1965, was performed. The results indicated that genetic analysis based on such groupings would be acceptable.

Peroxidase, esterase and L-amino-peptidase isozyme variations, in leaf, inflorescence and callus tissue were studied. The three tissues showed considerable variability in the three enzyme systems.

Variations were also observed between mature and young tissues in inflorescences, and between callus tissue grown in different media. In this connection a developmental study was made. Upon the removal of the inducing hormone, i.e. 2,4-D, callus tissue derived from young inflorescences from some of the parents and the hybrids, followed a developmental track giving roots, leaves and whole plants.

LITERATURE CITED

1. Akazawa, T., and Conn, E. E. 1958. The oxidation of reduced pyridine nucleotides by peroxidase. J. Biol. Chem. 232: 403-415.

2. Allison, A. C. 1959. Metabolic polymorphisms in mammals and their bearing on problems of biochemical genetics. Am. Nat. 93: 5-16.

3. Ashton, G. C. 1965. Cattle serum transferring: a balanced polymorphism. Genetics 52(5): 983-997.

4. _____ and Braden, A. W. H. 1961. Serum B-globulin polymorphism in mice. Austr. J. Biol. Sci. 14: 248-254.

5. _____ and Lampkin, G. H. 1965. Serum albumin and transferring polymorphism in African cattle. Nature, Lond. 205: 209-210.

6. Beckman, L. and John, F. M. 1964. Genetic control of aminopeptidases in Drosophila melanogaster. Hereditas 51: 221-230.

7. _____, Scandolios, J. G., and Brewbaker, J. B. 1964. Genetics of leucine amino-peptidase isozymes in maize. Genetics 50: 899-904.

8. Bhat, N. R. 1964. Potentialities of Sclerostachya fusca and Narenga porphyrocoma in sugar cane breeding. Ind. Jour. Sugar Cane Research Development 8(2): 121.

9. Boll, W. G. 1965. On the claimed adaptive nature of IAA oxidase and an effect of green light on IAA oxidase activity. Can. J. Botany 43: 884-892.

10. Bremer, G. 1923. A cytological investigation of some species and some hybrids within the genus Saccharum. Genetica 5: 97-148.

11. Burns, J. M. and Johnson, F. M. 1967. Esterase polymorphism in natural populations of a sulfur butterfly, Colias eurytheme. Science 156: 93-96.

12. Burton, G. W. 1959. Principles of breeding vegetatively propagated plants. Int. Soc. Sugar Cane Tech. 10th Congress, Hawaii, pp. 661-669.

13. Cann, J. R. and Goad, W. B. 1965. Theory of moving boundary electrophoresis of reversibly interacting systems. J. Biol. Chem. 240: 148-155.

14. Cann, J. R. and Goad, W. B. 1965. Theory of zone electrophoresis of reversibly interacting systems. J. Biol. Chem. 240: 1162-1164.

15. Chance, B. 1949. The properties of the enzyme-substrate compounds of horse-radish and lacto-peroxidase. Science 109: 204-208.

16. _____. 1957. Resistance to diseases in breeding varieties of sugar cane. Int. Symp. Genetics and Plant Breeding in South Asia. New Delhi, pp. 257-268.

17. Clausen, R. E. and Cameron, D. R. 1957. Inheritance in Nicoti-ana tabacum XXVIII. The cytogenetics of introgression. Proc. Nat. Acad. Sci., Washington 43: 908-913.

18. Dixon, M. and Webb, E. C. 1964. Enzymes. Academic Press, Inc. Publishers, New York, pp. 311-313.

19. Galston, A. W. and L. Y. Dalberg. 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. Am. J. Botany 41: 373-380.

20. George, P. 1953. The chemical nature of the second hydrogen peroxide compound formed by cytochrome C peroxidase and horse-radish peroxidase. Biochem. J. 54: 267-276.

21. Gerstel, D. U. 1946. Inheritance in Nicotiana tabacum XXI. The mechanism of chromosome substitution. Genetics 31: 421-427.

22. Grassl, Carl O. 1962. Problems and potentialities of Inter-intergeneric hybridication in sugar cane breeding programme. Proc. of the 11th Cong. The Int. Soc. Sugar Cane Technol. Mauritius, pp. 447-456.

23. Jacob, F. and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

24. Janaki-Ammal, E. K. 1940. Chromosome numbers in Sclerostachya fusca. Nature 145: 464.

25. _____. 1941. Intergeneric hybrids of Saccharum. Jour. Genet. 41: 217-253.

26. Jermyn, M. 1952. Multiple nature of the enzymes of Aspergillus oryzae and of horse-radish. Nature 169: 487-489.

27. Jermyn, M. and Thomas, R. 1954. Multiple components in horse-radish peroxidase. Biochem. J. 56: 631-639.

28. Klapper, M. H. and Hackett, D. P. 1965. Investigations on the multiple components of commercial horse-radish peroxidase. Biochem. Bio-Phys. Acta 96: 272-282.

29. Linsmaier, E. M. and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantanum 18: 100-127.

30. Markert, C. L. and Moller, F. 1959. Multiple forms of enzymes: tussie, ontogenetic, and species specific patterns. Proceedings of the National Academy of Science 45(5): 753-763.

31. McCune, D. 1960. Multiple peroxidases in corn. In Multiple Molecular Forms of Enzymes. Ann. N. Y. Acad. Sci. 94: 723-730.

32. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

33. Ockerse, R., Siegal, B. Z., and Galston, A. W. 1966. Hormone induced repression of a peroxidase isozyme in plant tissue. Science 151: 452-453.

34. Owen, J. A., Silberman, M. J., and Got, C. 1956. Detection of haemoglobin, haemoglobin-haptoglobin complexes and other electrophoresis. Nature 182: 1373.

35. Poulik, M. D. 1957. Starch gel electrophoresis in a discontinuous system of buffers. Nature 180: 1477-1479.

36. Raghavan, T. S. 1951. Some cytogenetic consideration. J. of Heredity 42(4): 199-206.

37. Scandalios, J. G. 1964. Tissue-specific isozyme variations in maize. J. of Heredity 55: 281-285.

38. Sears, E. R. 1956. The transfer of leaf rust resistance from aegilops umbellulata to wheat. Brookhaven Symposia in Bio. 9: 1-22.

39. Shannon, L. M., Kay, E., and Lew, J. V. 1966. Peroxidase isozymes from horse-radish roots. Isolation and physical properties. J. Biol. Chem. 241: 2166-2175.

40. Skoog, F., and Miller, C. O. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Exptl. Biol. 11:118-131.

41. Siegel, B. Z. and Galston, A. W. 1967. The isoperoxidases of Pisum sativum. Plant Physiol. 42: 221-226.

42. Smithies, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. 61: 629-641.

43. Steward, F. C. and Caplin, S. M. 1951. A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. Science 113: 518-520.

44. Steward, F. C. and Shantz, E. M. 1956. The chemical induction of growth in plant tissue culture. Warin and Wightman Conf. The chemistry and mode of action of plant growth substances. Butterworth's Scientific Publications, London, pp. 165-186.

45. Theorell, H. 1942. Crystalline peroxidase. Enzymologia 10: 250-252.

46. _____. 1951. The iron containing enzymes. B. catalases peroxidases "Hydroperoxidases". The Enzymes Vol. 11: Part 1.

47. Watson, J. D. and Crick, F. H. C. 1953. Molecular structure of nucleic acids. Nature 171: 737-738.

48. Yamazaki, I., Mason, H. S., and Piette, L. 1960. Identification by electron paramagnetic resonance spectroscopy of free radicals generated from substrates by peroxidase. J. Biol. Chem. 235: 2444-2449.